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Molecular structure and function analysis of bikunin on down-regulation of tumor necrosis factor-α expression in activated neutrophils

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Abstract

Objective: We performed a detailed molecular analysis of bikunin-mediated anti-inflammation (suppressive effect of cytokine release, MAP kinase activation, and nuclear translocation of NF-kB) using a truncated form of bikunin. *Materials and methods:* We obtained bikunin derivatives that contained *O*-glycoside-linked N-terminal glycopeptide (Bik-m1), *N*-glycoside-linked C-terminal tandem Kunitz domains (Bik-m2), bikunin lacking *O*-glycoside (Bik-c), asialo bikunin (Bik-a), bikunin lacking *N*-glycoside (Bik-n), and purified C-terminal Kunitz domain II (kII) of bikunin (HI-8). Enzyme-linked immunosorbent assay and Western blot were carried out to measure secreted TNF-α and MAP kinase activation. *Results:* We examined the TNF-α secretion in control and lipopolysaccharide (LPS)-treated neutrophils and did not see any changes of its protein levels in the cells pretreated with Bik-m1, Bik-m2, Bik-c, or HI-8. In all of the derivatives tested, only the derivatives that lacked *N*-glycoside side chain showed a significant suppression of TNF-α secretion by LPS. Only a small (21 amino acids) deletion of the N-terminal portion of bikunin (which corresponds to Bik-m2) abolished its suppressing activity of TNF-α secretion, thus suggesting that the N-terminal 21 amino acids play a critical role in anti-inflammation. Bik-m1 alone failed to show anti-inflammatory response. Bikunin failed to inhibit ionomycin-induced phosphorylation of MAP kinases. *Conclusion:* These data allow us to conclude that the cytokine expression was inhibited only by the *O*-glycoside-linked core protein without the *N*-glycoside side chain. Our results also suggest a possible role of bikunin for receptor-dependent MAP kinase activation.

Keywords: Bikunin; Proinflammatory cytokines; Neutrophils; Functional assay; Signal transduction

1. Introduction

Preterm delivery is thought to be mediated by proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) and other inflammatory mediators released from activated leukocytes [1]. Lipopolysaccharide (LPS), an inflammatory mediator, has been shown to activate neutrophils directly or indirectly through induction of MAPK-dependent NF-kB activation and expression of proinflammatory cytokine [2]. Because proinflammatory cytokines have been shown to activate neutrophils [3], inhibition of either neutrophil activation or proinflammatory cytokine production by some therapeutic agents might contribute to reduce inflammation-induced preterm delivery.

Bikunin, a well known Kunitz-type protease inhibitor (also known as urinary trypsin inhibitor [UTI] in Japan) purified from human urine, is clinically used for management of preterm delivery [4–6]. Several clinical studies

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demonstrated that bikunin has proved effective in patients developing preterm delivery. It is possible that intravaginal administered bikunin will reduce preterm delivery by inhibiting neutrophil activation. We have demonstrated previously that bikunin plays a critical role in anti-inflammation: it prevents the release of proinflammatory cytokines, activation of MAPK, phosphorylation of IkB-α, degradation of IkB-α, and nuclear translocation of NF-kB in macrophages stimulated with LPS [7,8]. Such inhibitory activities of bikunin on cell activation may contribute to reduce LPS-induced preterm delivery in in vivo experiments [9–13]. In the previous study, this possibility was examined in vitro [14]. However, the molecular networks underlying this event in bikunin-mediated anti-inflammation remain poorly understood.

Bikunin is produced as a light chain of inter-α-inhibitor (IαI) by liver cells [15]. The protein is heavily glycosylated at two sites, Ser10 and Asn45. It is a 40-kDa glycoprotein comprised of several structurally and functionally distinct domains [16]. This glycoprotein is composed of three domains: an N-terminal-domain with *O*-glycoside side chain (Ala1-Asp21), a middle-domain with *N*-glycoside side chain (Kunitz domain I [kI], Thr23-Trp77), and a C-terminal-domain (Kunitz domain II [kII], anti-tryptic activity, Ser78-Ala143) [16]. The anti-catalytic domain is covered by the C-terminal kII-domain. Bikunin has several conserved residues in the N-terminal first Kunitz domain (kI) and the C-terminal second Kunitz domain (kII), including six cysteines. Both domains are predicted to have a similar structure, presumably maintained by disulfide bonds between the conserved cysteines [16].

It was shown that bikunin inhibited LPS-mediated increases in intracellular calcium (Ca^{2+}) [17]. LPS recognition requires its binding protein (LBP), CD14, and MD-2, which is directly involved in ligand binding and subsequent receptor activation [18]. Then, LPS activates neutrophil signal transduction in a receptor-dependent manner, which is related to LPS-mediated increases in Ca^{2+}. On the other hand, Ca^{2+}-sensitive ionophore ionomycin directly causes changes in cytosolic Ca^{2+} level, and also activates MAP kinases in a receptor-independent manner [19]. It is unclear whether bikunin-mediated suppression of MAP kinase activation is a receptor-dependent.

In the present study, we performed a detailed molecular analysis of bikunin-mediated anti-inflammation using a truncated form of bikunin, to investigate whether the effects of bikunin were mediated by its Kunitz structure or glycoside side chain. We finally examined the suppressive effect of bikunin on LPS- or ionomycin-induced changes in both p38 and p42/44 MAP kinase activation.

2. Experimental procedures

2.1. Materials

Bikunin was purified to homogeneity from human urine. A highly purified preparation of bikunin was kindly supplied by Mochida Pharmaceutical (Tokyo, Japan). The kII (HI-8) was purified from bikunin as previously described [16]. Metalloendopeptidase (EC 3.4.2.4), chondroitinase ABC lyase (EC 4.2.2.4), neuraminidase (EC 3.2.1.18), endo-β-*N*-acetylglucosaminidase F2 (Endo F2; EC 3.2.1.92), and hyaluronidase were purchased from Seikagaku Kogyo (Tokyo, Japan). Trypsin (EC 3.4.21.4) was purchased from Sigma–Aldrich, Japan (Tokyo). Sephadex G-100, Sephacryl S-200HR, and Con A-Sepharose were purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). All experiments were performed with LPS from Escherichia coli 0111:B4 endotoxin (Sigma–Aldrich, Tokyo, Japan). For the generation of stock solutions, all reagents were dissolved in endotoxin-free water (Sigma–Aldrich). NF-kB p50 was purchased from Cell Signaling Technologies (Beverly, MA) and Santa Cruz Biotechnology (Santa Cruz, CA). HRP-labeled anti-rabbit/goat Abs, and ECL reagents were purchased from Bio-Rad. Custom-mixed Abs and columns for neutrophil isolation were purchased from Stem Cell Technologies (Vancouver, BC, Canada). Ca^{2+}-selective ionophore ionomycin was from Sigma. All other chemicals were of reagent grade or better and were purchased from major suppliers.

2.2. Preparation of bikunin derivatives

Preparation of bikunin derivatives was performed as previously described [16]. Bikunin was digested with chondroitinase ABC (Bik-c), metalloendopeptidase (Bik-m1 and Bik-m2), neuraminidase (Bik-a), or endo F2 (Bik-n). The structure of bikunin derivatives was analyzed by SDS–PAGE, N-terminal sequence, and amino acid analysis, content of acidic, neutral and amino sugars, and anti-trypsin activity (see Fig. 1 and Ref. [16]). The purity of bikunin derivatives were confirmed by SDS–PAGE and Western blot [16].

2.3. Cells and culture conditions

Bone marrow neutrophils were isolated as described previously [20]. The cell suspension was incubated with primary Abs specific for cell surface markers F4/80, CD4, CD45R, CD5, and TER119. This custom cocktail is specific for T and B cells, RBC, monocytes, and macrophages. Anti-biotin tetrameric Ab complexes were added and rotating. Colloidal magnetic dextran iron particles were added to the suspension and incubated and rotating. The entire cell suspension was then placed into a column, surrounded by a magnet. Neutrophils were obtained by negative selection methods. Neutrophil purity, as determined by Wright's stained cytospin preparations, was greater than 97%. Neutrophils (1×10^6/ml) were cultured in RPMI 1640, 10% FCS, penicillin, and streptomycin. In each experiment bikunin was added to the cell cultures 1 h before stimulation with LPS. To analyze the inhibitory effect of bikunin on cytokine release, neutrophils (2×10^6 cells/well) were incubated with bikunin, and the